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## TECHNICAL MANUSCRIPT 569

### SOME CHARACTERISTICS OF RIBOFLAVIN CHEMILUMINESCENCE

Richard D. Towner  
Harold A. Neufeld  
Philip B. Shevlin

DECEMBER 1969

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SOME CHARACTERISTICS OF RIBOFLAVIN CHEMILUMINESCENCE

Richard D. Towner

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Physical Science Division  
BIOLOGICAL SCIENCES LABORATORIES

Project 1B662706A071

December 1969

ABSTRACT

Data are presented that describe some characteristics of riboflavin chemiluminescence in the dark in the presence of hydrogen peroxide and osmium trichloride. The reaction, in terms of the light intensity produced, is affected by type of buffer, pH, and concentration of reactants. Light intensity is directly proportional to the concentration of riboflavin, and a 1,000-fold increase in relative light intensity is produced when osmium is present. The optimum pH for the reaction in phosphate buffer is 8.0 and in sodium hydroxide, 12. Spectral studies of the riboflavin chemiluminescent reaction show that the emitting species has a maximum intensity of emission at a wavelength different from the fluorescence maximum of riboflavin. The observed 495-m $\mu$  maximum is similar to the maximum observed for the luminescent bacterium, Achromobacter fischeri.

CONTENTS

Abstract . . . . .	2
I. INTRODUCTION . . . . .	5
II. MATERIALS AND METHODS . . . . .	6
III. RESULTS AND DISCUSSION . . . . .	9
Literature Cited . . . . .	17
Distribution List . . . . .	19
DD Form 1473 . . . . .	21

FIGURES

1. Schematic Drawing of the EMI Phototube Apparatus . . . . .	7
2. Effect of Buffer and pH on Riboflavin Chemiluminescence . . . . .	10
3. Effect of Riboflavin Concentration at pH 8.0 . . . . .	10
4. Effect of $\text{OsCl}_3 \cdot 3\text{H}_2\text{O}$ Concentration on Riboflavin Chemiluminescence at pH 8.0 . . . . .	11
5. Effect of Hydrogen Peroxide Concentration at pH 8.0 in Phosphate Buffer . . . . .	11
6. Inverse Plot of Osmium Concentration and Light Intensity at pH 8.0 . . . . .	12
7. A Comparison of Chemiluminescent Reaction Time with Disappearance of Riboflavin Absorption . . . . .	12
8. Absorption Spectrum of Riboflavin Before and After Chemiluminescent Reaction . . . . .	13
9. Fluorescence Spectra of Riboflavin . . . . .	13
10. Emission Spectrum of the Chemiluminescent Reaction of Riboflavin and the <i>in vitro</i> System of <i>Achromobacter fischeri</i> . . .	15
11. Effect of Osmium Trichloride Catalyst on Riboflavin Chemiluminescence at Different pH Values . . . . .	16

## I. INTRODUCTION\*

Only a few of many bioluminescent species of life have been investigated thoroughly enough to demonstrate in vitro reactions of light emission with complete characterization of the individual constituents of the reaction. Three well-known bioluminescent systems studied in vitro are those of Cypridina, fireflies, and bacteria such as Achromobacter fischeri and Photobacterium phosphoreum. The reactions involved have been summarized by Johnson, Sie, and Haneda.<sup>1</sup> In the bacterial in vitro system, reduced flavine mononucleotide ( $\text{FMNH}_2$ ) is oxidized in the presence of bacterial luciferase, a long-chain aldehyde, and oxygen to yield flavine mononucleotide (FMN), other products, water, and light.<sup>1</sup>

McElroy and Green<sup>2</sup> suggested the possibility that peroxidation of an aldehyde molecule was the source of the excitation energy and proposed that one  $\text{FMNH}_2$  molecule combined with the aldehyde and that a second  $\text{FMNH}_2$  molecule combined with oxygen to form a peroxide. They presumed that the organic peroxide acted as an oxidant for the aldehyde- $\text{FMNH}_2$  compound to yield a highly excited molecule that emitted light.

Johnson<sup>3</sup> states that no activating aldehyde has yet been isolated from luminescent bacteria. Bacterial luciferin may be considered to have been synthesized if  $\text{FMNH}_2$  is considered the light-emitter of the bacterial system.<sup>3</sup> The structure of firefly luciferin was proven by synthesis in 1961 by White et al.<sup>4</sup> Cypridina luciferin was crystallized in 1957 by Shimomura, Goto, and Hirata,<sup>5</sup> and its structure was established by Kishi et al.<sup>6</sup> in 1965.

Hastings and Gibson<sup>7</sup> have reported evidence of intermediates in the bioluminescent oxidation of  $\text{FMNH}_2$ , and Terpstra<sup>8</sup> found evidence of a precursor compound in studying the light-emitting molecule in P. phosphoreum.

The recent work of Hastings and Gibson<sup>7</sup> and others on bacterial luminescence, while shedding much light on the mechanism of the enzyme-catalyzed reaction, did not produce evidence concerning the nature of the emitting group. Strehler and Shoup,<sup>9</sup> in their paper on riboflavin luminescence, produced data that indicated that the maximum wavelength of riboflavin chemiluminescence was identical to the fluorescence maximum for riboflavin. We present data in this paper that place the maximum wavelength of riboflavin chemiluminescence not at 535 m $\mu$ , which is the fluorescence maximum, but at 495 m $\mu$ , which is close to the maximum wavelength of the emission from several luminescent bacteria.

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## II. MATERIALS AND METHODS

All reagents were prepared fresh daily. Riboflavin was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, and from Merck & Company, Inc., Rahway, New Jersey. Solutions such as buffers, riboflavin standards, and osmium chloride dilutions were prepared with demineralized distilled water, which was made by passing distilled water through a Barnstead Bantam ion-exchange demineralizer. Riboflavin standard solutions were prepared by dissolving 5 mg of riboflavin in 100 ml of water. More concentrated riboflavin solutions were used in some experiments.

Reagent grade 30% H<sub>2</sub>O<sub>2</sub>\* was stored in the cold (3 to 5 C). When 30% H<sub>2</sub>O<sub>2</sub> was used, the disodium salt of ethylenediaminetetraacetic acid (EDTA) was added to it to yield a final concentration of 1 mg EDTA/ml. When these 30% H<sub>2</sub>O<sub>2</sub> solutions containing EDTA were diluted, the dilutions were made with demineralized distilled water that also contained 1 mg EDTA/ml. Previous work in this laboratory showed that EDTA stabilized H<sub>2</sub>O<sub>2</sub> solutions and insured reproducibility of data.<sup>10</sup>

Stock OsCl<sub>3</sub>·3H<sub>2</sub>O solutions were prepared by dissolving 10 mg of OsCl<sub>3</sub>·3H<sub>2</sub>O\*\* in 10 ml of demineralized distilled water or in 0.1 M phosphate buffer (pH 8.0). Borate, phosphate, and tris buffers were used for studying the effect of pH and buffers on luminescence. The reaction was also carried out in sodium hydroxide.

The procedure for light measurement involved the rapid recording of a flash of light or, in some instances, the recording of a steady light emission. The phototube apparatus used for light measurement detected the light produced during the reaction of riboflavin, H<sub>2</sub>O<sub>2</sub>, and OsCl<sub>3</sub>·3H<sub>2</sub>O. Reactions were initiated by injecting 0.1 ml of H<sub>2</sub>O<sub>2</sub> solutions of various concentrations. Individual reactants, exclusive of H<sub>2</sub>O<sub>2</sub>, were pipetted into a Pyrex test tube (10 by 75 mm) and mixed with a Vortex mixer briefly before placing into the lighttight compartment of the apparatus. After the tube containing the reaction mixture was placed in the chamber, it was covered by a metal cap containing a rubber serum stopper through which injections were made. The apparatus is designed so that an external sliding shutter facilitates the exposure of the phototube to the reaction tube in the dark just before injection. An EMI phototube (model 9558B) was used to detect light emission. Amplification was provided by a Sanborn preamplifier, model 150-1500. Data were recorded on a Sanborn 151 recorder. High voltage was provided by a Sanborn high-voltage supply unit. The light-measuring apparatus is shown in Figure 1.

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\* Fisher Scientific Co., Fair Lawn, New Jersey.

\*\* Alfa Inorganics, Inc., Beverly, Massachusetts.

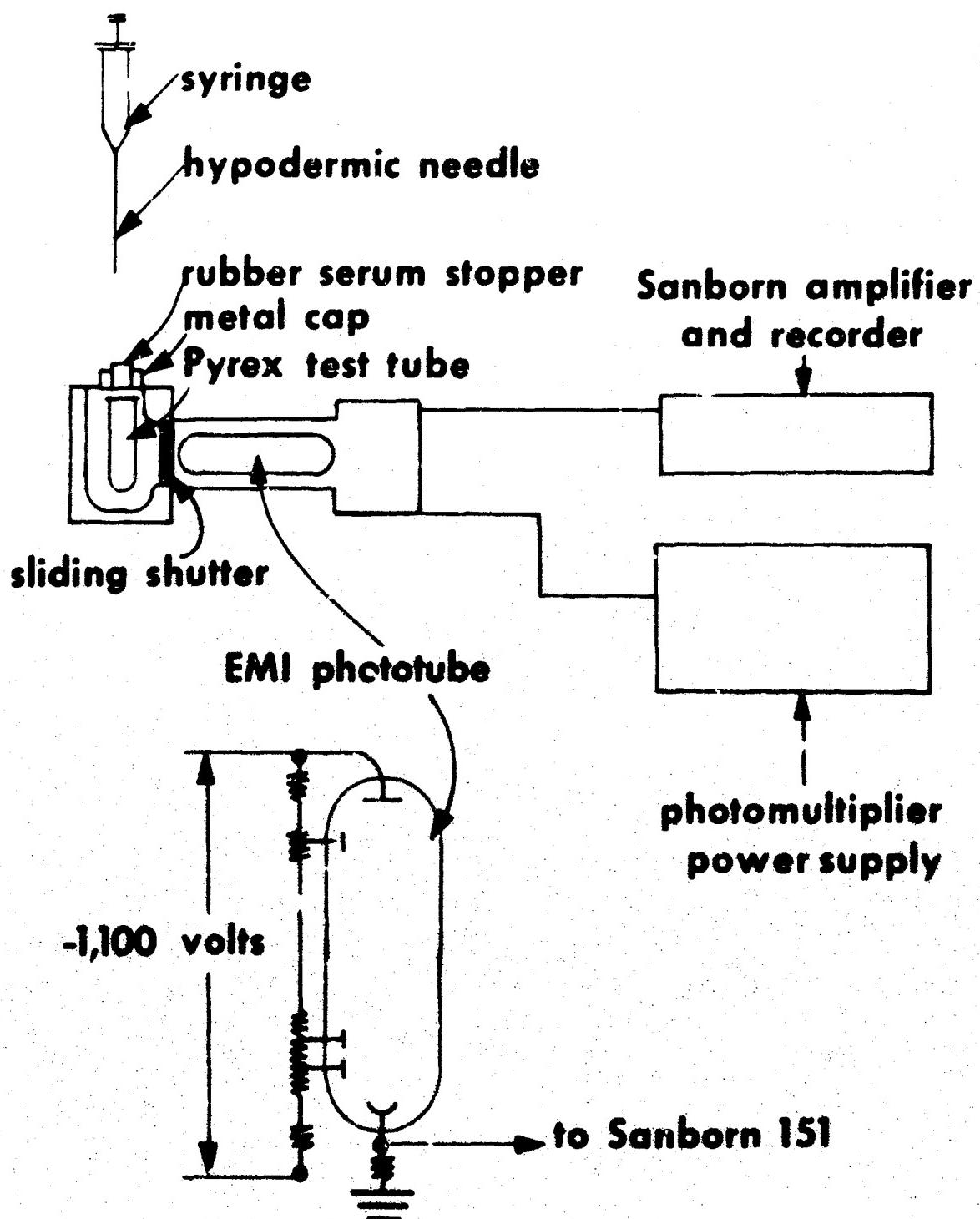


FIGURE 1. Schematic Drawing of the EMI Phototube Apparatus.

Absorption studies were carried out in a Beckman DU spectrophotometer. Matched quartz cuvettes were used, and special precaution was taken to insure that solutions were free of bubbles during absorption work. Extra care was also necessary for preparation of the reaction test tubes. All Pyrex test tubes were washed in distilled water after use and then placed in concentrated nitric acid overnight. Acid-washed tubes were further washed with copious amounts of distilled water and finally with several rinses of demineralized distilled water before oven drying.

Additional studies of fluorescent and luminescent spectra were carried out in a modified Aminco-Bowman spectrofluorometer. The Aminco-Bowman sample chamber was changed to accommodate a Pyrex test tube (6 by 1.4 cm) by removal of the cuvette housing. The chamber was made lighttight with black electrical tape. A test tube was placed in the center of the chamber by fitting it on a rubber serum stopper that was fitted into a centered hole drilled in a metal cover for the chamber. No slits were used in the sample chamber so that maximum light could be obtained from the low light level chemiluminescent reaction. This permitted peroxide to be injected into the sample in the dark and the resulting emission spectra to be measured. A IP28 photomultiplier tube and a high-voltage power supply were employed and data were recorded on a Varian recorder (model G-14). The modified Aminco-Bowman sample chamber was checked for accuracy by the use of light emission produced by luminol in alkaline solution containing potassium persulfate ( $K_2S_2O_8$ ) and  $H_2O_2$ , and also by luminol in dimethyl sulfoxide. Alkaline luminol had a maximum emission at 435  $\mu\text{m}$ , and luminol in dimethyl sulfoxide had a maximum emission at 480  $\mu\text{m}$ . These results are in close agreement with reported values of luminol chemiluminescence.<sup>11</sup>

All of the spectral data presented in the paper are corrected for the spectral sensitivity of the IP28 photomultiplier tube.<sup>12</sup> Fluorescent spectra of riboflavin and chemiluminescent emission spectra of luminol were obtained both by repetitive scanning and by stepwise rotation of the monochromator wavelength drum. The chemiluminescent emission spectrum of the riboflavin reaction was obtained in a stepwise manner by injection of peroxide into reactant mixtures over a range of emission wavelength settings.

### III. RESULTS AND DISCUSSION

Figure 2 shows the effect of various buffers on riboflavin chemiluminescence. In this experiment, the concentrations of riboflavin,  $\text{OsCl}_3 \cdot 3\text{H}_2\text{O}$ , and  $\text{H}_2\text{O}_2$  were held constant with only the type of buffer and the pH varied. These data show that both the pH and the particular buffer influenced the amount of light produced. Maximum light intensity occurred in NaOH at pH 12. Somewhat lower intensities were observed with borate and phosphate buffers, while the least amount of light was obtained with tris buffer. Although maximum light intensity was always obtained at pH 12 in NaOH, some of the data to be discussed here will be based on experiments run at pH 8.0 in phosphate buffer in order to approach the physiological pH of bacterial luminescence and, also, to avoid the effects of the rather violent reactions that often occurred at the higher pH. In these cases the resulting foaming often interfered with accurate readings of light intensity.

Figure 3 demonstrates that the reaction velocity is first order with respect to riboflavin concentration. The linear relationship between light intensity and riboflavin concentration was obtained whether the riboflavin concentration was greater or less than that of the  $\text{OsCl}_3 \cdot 3\text{H}_2\text{O}$ . Moreover, the linear relationship between light intensity and riboflavin concentration was obtained whether flash height or total integrated light emission was plotted against the riboflavin concentration.

Nonlinear relationships were obtained when the intensity of the light was plotted against either the  $\text{OsCl}_3 \cdot 3\text{H}_2\text{O}$  or  $\text{H}_2\text{O}_2$  concentration. These data are illustrated in Figures 4 and 5. However, if a reciprocal plot of light intensity and osmium concentration is made, a linear relationship results as shown in Figure 6. This relationship is also linear when the  $\text{H}_2\text{O}_2$  concentration is plotted in the same manner.

The data in Figure 7 demonstrate that all of the riboflavin absorption at  $371 \mu\text{m}$  disappeared by the time the luminescent intensity reached zero. Figure 8 shows that total destruction of the riboflavin chromophore occurs during chemiluminescence. Strehler and Shoup<sup>9</sup> suggest that oxidation produces luminescence at about the same wavelength as riboflavin fluorescence. In an attempt to confirm this we compared the fluorescent spectrum of riboflavin with the emission spectrum of the chemiluminescent reaction. The fluorescent spectra were obtained under similar conditions as the chemiluminescent spectra except that  $\text{H}_2\text{O}_2$  was absent. Results indicate that the presence of  $\text{OsCl}_3 \cdot 3\text{H}_2\text{O}$  in the system does not affect the maximum wavelength of riboflavin fluorescence but does quench the amount of light produced to some extent. Figure 9 shows that the fluorescent spectrum of riboflavin excited at  $371 \mu\text{m}$  has a maximum at  $535 \mu\text{m}$ . This is in agreement with the fluorescent spectrum reported by Yagi and Okuda.<sup>13</sup>

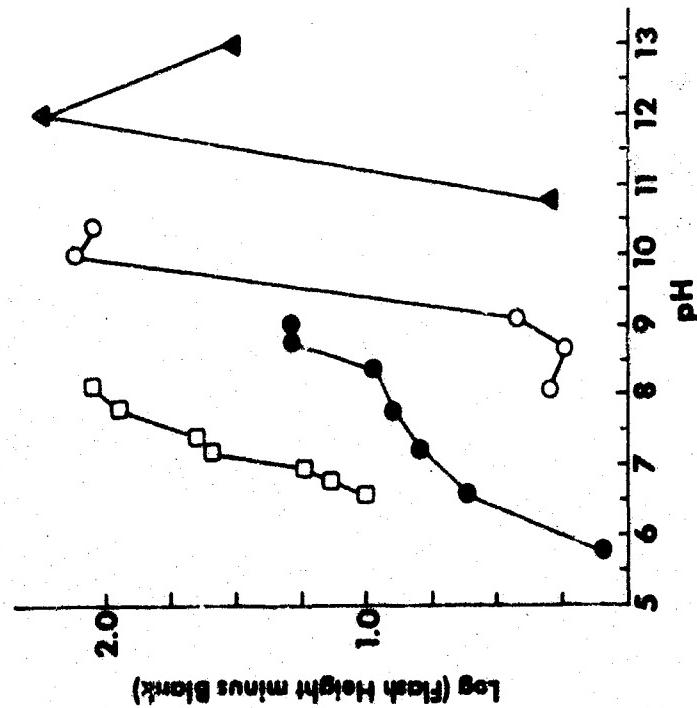


FIGURE 2. Effect of Buffer and pH on Riboflavin Cleavage. A 0.6-ml reaction mixture contained  $7.1 \times 10^{-5}$  M riboflavin,  $4.75 \times 10^{-6}$  M  $\text{OscCl}_3 \cdot 3\text{H}_2\text{O}$ ,  $1.47 \text{ M H}_2\text{O}_2$ , and 0.1 N buffer or NaOH. Phosphate buffer (□); tris buffer (○); borate buffer (●); sodium hydroxide (▲).

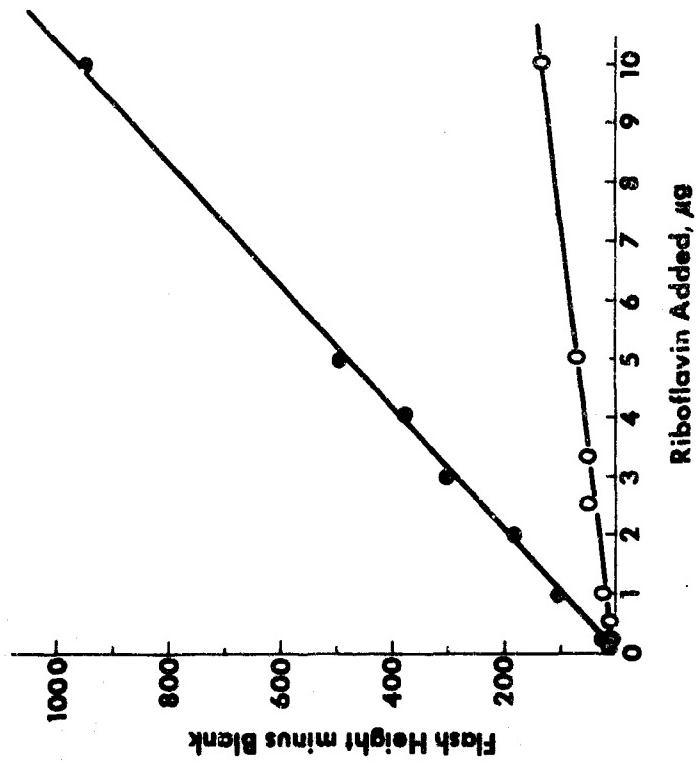


FIGURE 3. Effect of Riboflavin Concentration at pH 8.0. A reaction volume of 0.6 ml contained  $1.9 \times 10^{-4}$  M  $\text{OscCl}_3 \cdot 3\text{H}_2\text{O}$ ,  $1.47 \text{ M H}_2\text{O}_2$ , 0.1 N phosphate buffer (pH 8.0) and variable amounts of riboflavin ( $4.43 \times 10^{-5}$  M to  $4.43 \times 10^{-7}$  M) (●). The effect of riboflavin concentration is shown under the same conditions except that the concentration of  $\text{OscCl}_3 \cdot 3\text{H}_2\text{O}$  was  $9.50 \times 10^{-6}$  M (○).

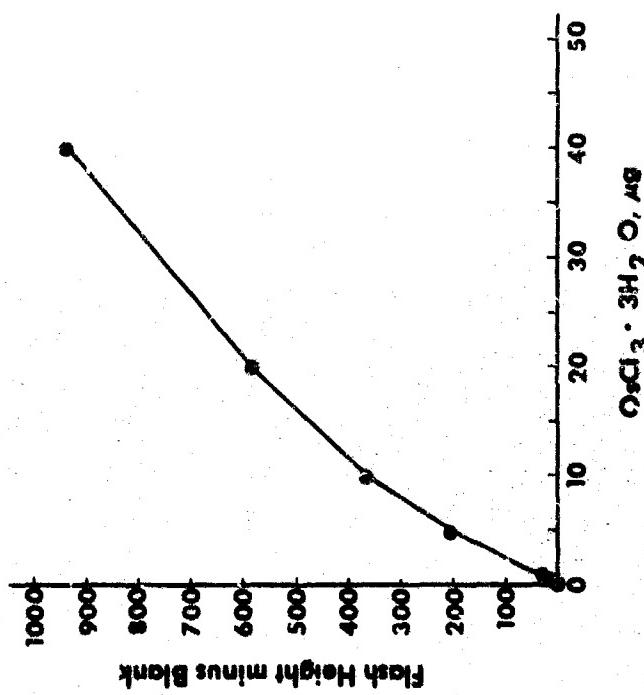


FIGURE 4. Effect of OsCl<sub>3</sub> · 3H<sub>2</sub>O Concentration on Riboflavin Chemiluminescence at pH 8.0. A 0.6-ml reaction mixture contained  $1.12 \times 10^{-5}$  M riboflavin,  $1.9 \times 10^{-4}$  M OsCl<sub>3</sub> · 3H<sub>2</sub>O, 0.1 M phosphate buffer (pH 8.0), and variable amounts of H<sub>2</sub>O<sub>2</sub> ( $4.9 \times 10^{-3}$  M to  $1.47$  M).

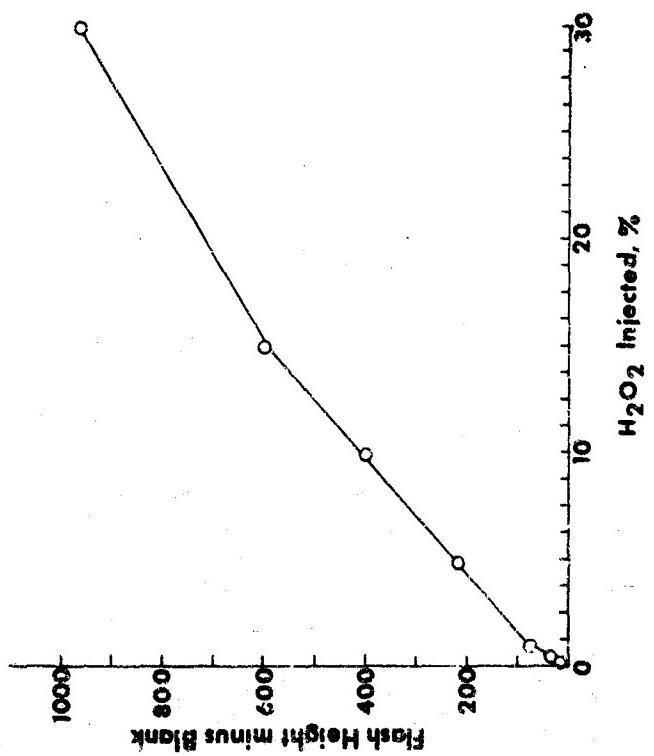


FIGURE 5. Effect of Hydrogen Peroxide Concentration at pH 8.0 in Phosphate Buffer. A 0.6-ml reaction mixture contained  $4.43 \times 10^{-5}$  M riboflavin,  $1.9 \times 10^{-4}$  M OsCl<sub>3</sub> · 3H<sub>2</sub>O, 0.1 M phosphate buffer (pH 8.0), and variable amounts of H<sub>2</sub>O<sub>2</sub> ( $4.9 \times 10^{-3}$  M to  $1.47$  M).

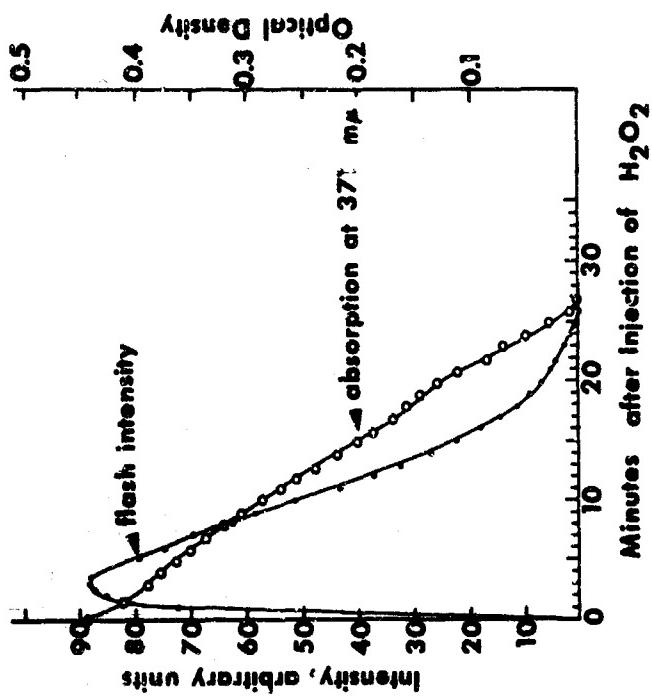


FIGURE 7. A Comparison of Chemiluminescent Reaction Time with Disappearance of Riboflavin Absorption. The absorption spectrum and chemiluminescent reaction were carried out in a reaction volume of 3.2 ml. This contained  $4.16 \times 10^{-5}$  M riboflavin,  $1.78 \times 10^{-6}$  M  $\text{OsCl}_3 \cdot 3\text{H}_2\text{O}$ ,  $9.19 \times 10^{-3}$  M  $\text{H}_2\text{O}_2$ , and 0.1 M phosphate buffer, pH 8.0.

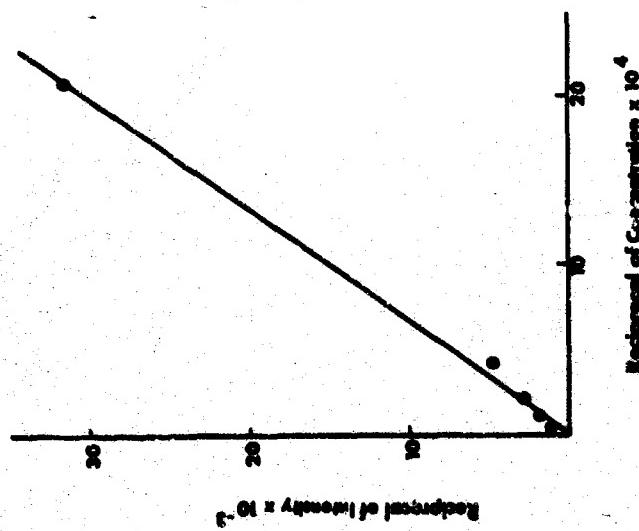


FIGURE 6. Inverse Plot of Osmium Concentration and Light Intensity at pH 8.0. The reciprocals of light intensities shown in Figure 4 are plotted against the reciprocals of osmium concentration.

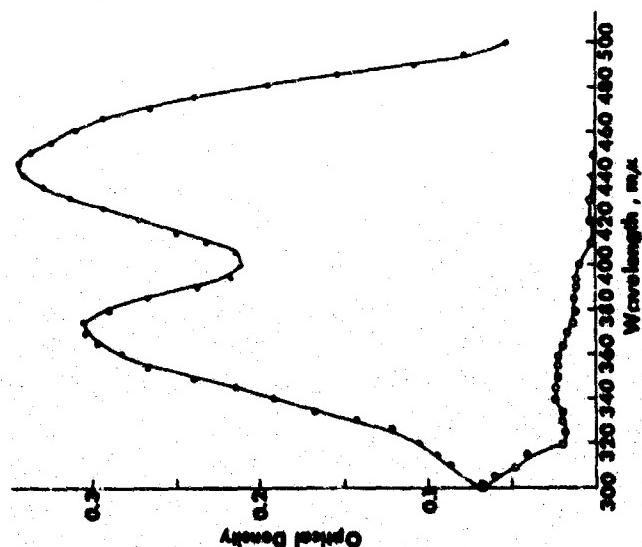


FIGURE 8. Absorption Spectrum of Riboflavin Before and After Chemiluminescent Reaction. The reaction mixture of 4.0 ml contained  $3.3 \times 10^{-5}$  M riboflavin,  $2.85 \times 10^{-6}$  M  $\text{OsCl}_3 \cdot 3\text{H}_2\text{O}$ , 0.441 M  $\text{H}_2\text{O}_2$ , 0.1 M phosphate buffer, and demineralized  $\text{H}_2\text{O}$ . The black contained the same constituents except riboflavin. Absorption of riboflavin (O); absorption of reaction products (●).

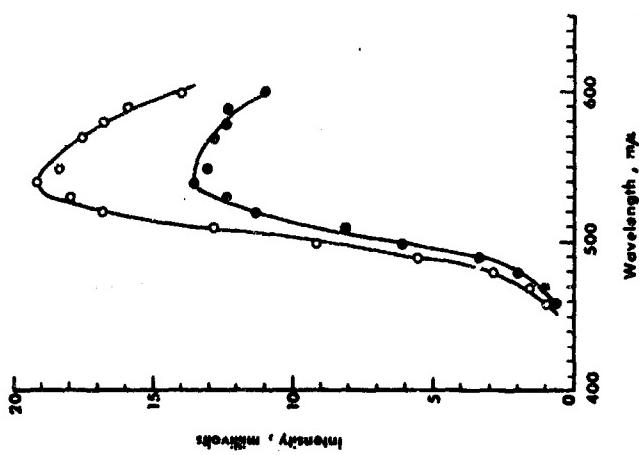


FIGURE 9. Fluorescence Spectra of Riboflavin. A  $4.44 \times 10^{-4}$  M riboflavin solution was activated at 371 mμ (O). The fluorescent spectrum of the same concentration of riboflavin with  $7.58 \times 10^{-5}$  M  $\text{OsCl}_3 \cdot 3\text{H}_2\text{O}$  present is also shown (●).

The wavelength of maximum emission of the chemiluminescent reaction was 495 m $\mu$ . This spectrum is close or similar to the emission spectrum of A. fischeri.<sup>14</sup> The emission spectrum of partially purified A. fischeri luciferase was obtained in an in vitro system by injection of reduced nicotine adenine dinucleotide into the partially pure enzyme system containing dodecyl aldehyde. The emission spectrum of the riboflavin reaction is compared with the emission spectrum of the A. fischeri in vitro system in Figure 10.

Figure 11 demonstrates that optimum light production is obtained when riboflavin, OsCl<sub>3</sub>·3H<sub>2</sub>O, and H<sub>2</sub>O<sub>2</sub> are all present. The reaction without osmium present produces only about one-thousandth the amount of light.

At the present time we can only speculate regarding the nature of the emitter in the reaction. Paper chromatography of irradiated riboflavin has been reported to yield decomposition products such as lumichrome which fluoresced at 490 m $\mu$ .<sup>15</sup> Hais and Pecakova also report that exposure of riboflavin and lumichrome to H<sub>2</sub>O<sub>2</sub> in alkaline solution yielded several more products, several of which fluoresced in the blue range.

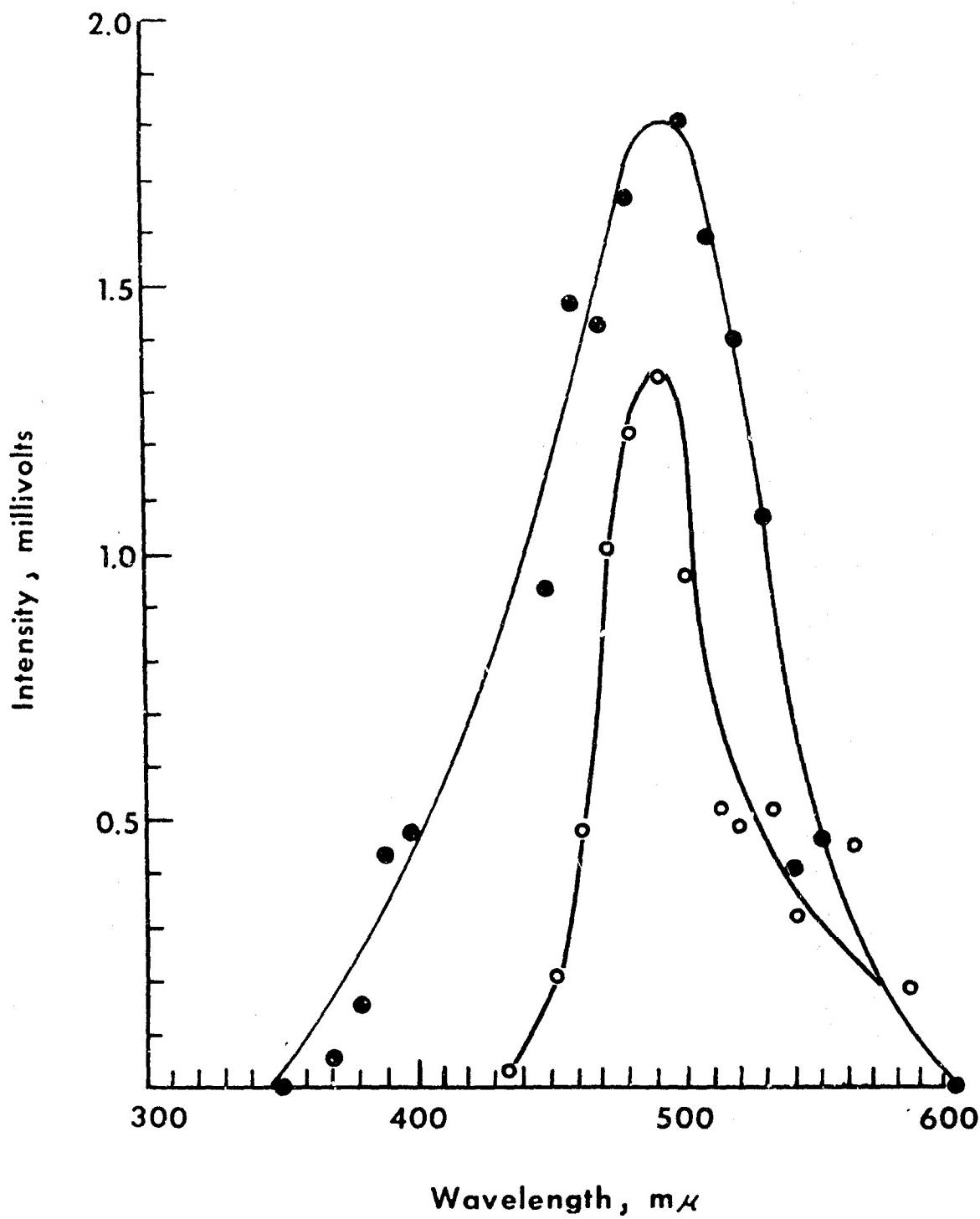


FIGURE 10. Emission Spectrum of the Chemiluminescent Reaction of Riboflavin and the in vitro System of *Achromobacter fischeri*. A reaction volume of 1.3 ml contained  $1.01 \times 10^{-4}$  M riboflavin,  $1.75 \times 10^{-4}$  M  $\text{OsCl}_3 \cdot 3\text{H}_2\text{O}$ , and 0.679 M  $\text{H}_2\text{O}_2$ . The spectrum represents a number of reaction emissions at intermittent wavelengths using the same concentration of reactants for each point (●). The in vitro system contained 0.1 ml of partially purified luciferase that mixed with dodecyl aldehyde in a 10:1 enzyme/aldehyde ratio. The reaction was initiated by injecting 0.1 ml of reduced nicotine adenine dinucleotide (0.1 mg/ml) in pH 8 phosphate buffer. The spectrum of the in vitro reaction also represents a number of reaction emissions at intermittent wavelengths (○).

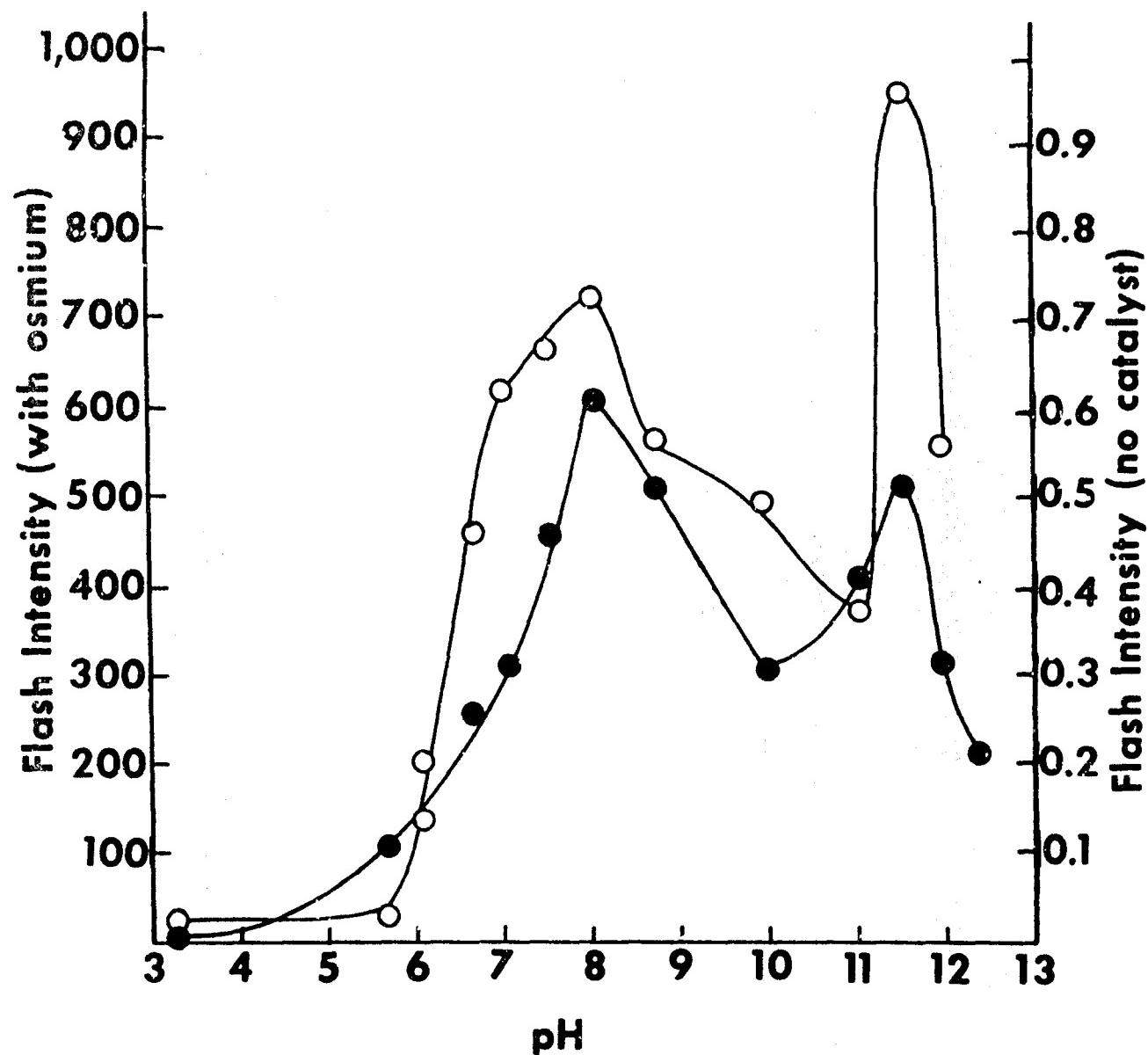


FIGURE 11. Effect of Osmium Trichloride Catalyst on Riboflavin Chemiluminescence at Different pH Values. A reaction volume of 0.8 ml contained  $4.21 \times 10^{-5}$  M riboflavin,  $1.27 \times 10^{-4}$  M  $\text{OsCl}_3 \cdot 3\text{H}_2\text{O}$ , 2.09 M  $\text{H}_2\text{O}_2$ , and a series of phosphate buffers adjusted at pH extremes with either hydrochloric acid or NaOH. Intensity with no osmium trichloride (●); intensity with osmium trichloride present (○).

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13. ABSTRACT Data are presented that describe some characteristics of riboflavin chemiluminescence in the dark in the presence of hydrogen peroxide and osmium trichloride. The reaction, in terms of the light intensity produced, is affected by type of buffer, pH, and concentration of reactants. Light intensity is directly proportional to the concentration of riboflavin, and a 1,000-fold increase in relative light intensity is produced when osmium is present. The optimum pH for the reaction in phosphate buffer is 8.0 and in sodium hydroxide, 12. Spectral studies of the riboflavin chemiluminescent reaction show that the emitting species has a maximum intensity of emission at a wavelength different from the fluorescence maximum of riboflavin. The observed 495-m $\mu$ maximum is similar to the maximum observed for the luminescent bacterium, <u>Achromobacter fischeri</u> .	
14. Key Words Chemiluminescence Light emission Luciferase Riboflavin Osmium trichloride Hydrogen peroxide <u>Achromobacter fischeri</u>	